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RESEARCH PAPER

Optimization of embryo rescue technique for development of hybrid plants in stenospermic grapes

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Abstract

The development of seedless cultivars is a primary goal in grapevine breeding. Since grapes are stenospermic and tend to abort their embryos before development, traditional breeding methods often yield seedless cultivars at a low frequency. Therefore, embryo rescue has emerged as a promising approach for creating seedless grape cultivars. This study aimed to optimize the ideal sampling time and protocol for efficient embryo rescue in grapes. Ovules from immature berries collected at various days after pollination (20DAP, 30DAP, 40DAP, and 50DAP) were cultured on Nitsch and Nitsch (NN) medium with different concentrations of Benzyl aminopurine (BAP) (0.1, 0.2, and 0.5 mg/L) and Activated Charcoal (AC) (1.5, 2, and 2.5 g/L). Several parameters were assessed, including maximum ovule growth, the percentage of enlarged ovules, percentage of collapsed ovules, callus formation percentage, and embryo germination percentage. The study's results indicated that berries collected at 40 DAP yielded the best outcomes across all parameters. Regarding treatments, the most favorable results were achieved when ovules were cultured on NN medium supplemented with 0.5 mg/L BAP and 2 g/L AC. In conclusion, the study underscores the significance of choosing the right sampling time and treatments to ensure efficient embryo rescue in grapes. The protocol standardized from this research is recommended for effectively rescuing embryos and developing seedless hybrids.

Keywords

Activated charcoal, Benzyl aminopurine, Embryo rescue, Grape, Nitsch, Ovule culture

Introduction

Seedless grapes, which are in high demand among consumers, unfortunately, tend to be more vulnerable to various diseases. The extensive use of different fungicides not only raises health concerns but also escalates costs. Therefore, there is a critical need to develop disease-resistant seedless grape varieties. This would not only meet consumer demand but also significantly decrease the need for costly fungicide applications, promoting both consumer satisfaction and cost efficiency. The use of the embryo rescue technique indeed holds great promise in achieving disease-resistant seedless grape varieties. Conventional breeding methods face significant challenges in grapes due to stenospermic behavior, making the embryo rescue technique a valuable and likely solution for

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overcoming these limitations and developing the desired disease-resistant seedless grape cultivars. Stenospermocarpy, which involves the abortion of grape embryos before they can fully develop, results from the abnormal development of the ovule and integument. Several factors play an important role in influencing the phenotypic expression of stenospermic grapes. These factors include seed trace size, ovule abortion, seed coat hardness, and environmental conditions (Cui et al. 2017; Conner et al. 2018). Abnormal endosperm can be obtained from aborted seed 30 days after blooming. Malabarba et al. (2018) reported that *VviAGL11* could control endotesta formation in seeded grapes during seed development stage. However, in the case of stenospermic grapes, this control mechanism does not seem to function effectively, which may contribute to the abortion that occurs in seedless grape varieties. Another reason for embryo abortion is the alteration of various endogenous hormones. Gibberellin is crucial for seed development, and the external application of gibberellic acid can induce seedlessness in grapes (Agüero et al. 2000). Costantini et al. (2008) also reported the role of GA_3 in seedlessness behavior in grapes and its interaction with auxins and other hormones. GA_3 treatment at full bloom significantly enhances gibberellin signaling and activates various phytohormone signaling pathway genes in ovules, but has a lesser effect on ovaries, indicating that GA_3 directly influences seed development (Nishiyama et al. 2022). Regarding seedless grape breeding, using embryos is a viable approach, as their totipotent cells can be cultured at the appropriate stage. Zygotic embryogenesis and embryo rescue techniques have proven to be effective strategies for developing seedless grape varieties (Roichev et al. 2007).

Many factors play an important role in the success of embryo rescue technique, like sampling time, parental combination, exogenous application of growth hormones and media type, etc. Determining the optimal sampling time is a critical factor in the success of the embryo rescue technique, especially when developing seedless grapes (Li et al. 2014). If sampling is too early, embryos may not have reached a suitable developmental stage, making them challenging to rescue and culture effectively. On the other hand, if sampling is too late, the embryos may have already aborted, rendering the rescue process futile. Therefore, precise timing and careful observation of grapevine development stages are essential to maximize the chances of successful embryo rescue. (Li et al. 2004). The choice of genotypes used in the genetic improvement programs of seedless grapes also has a significant effect on the efficiency of embryo rescue (Puglisi et al. 2022). Composition of the culture medium, including the concentrations of plant growth regulators, plays a pivotal role in the efficiency of embryo rescue (Razi et al. 2013). Media composition, pollen parent and year of cross also have great effect on embryo development and cultured ovules recovery (Liu et al. 2008). Furthermore, plant growth regulators (PGRs) were important and effective for the embryo rescue technique and have been widely used. PGRs were added to the culture medium to increase the number of larger embryos and enhance the number of plants recovered (Ebadi et al. 2016). By keeping in view, the importance of these factors, the study aims to determine optimal sampling time and hormonal concentrations for achieving efficient embryo rescue in grapes.

Materials and methods

Collection of berries

Berries from six local grape genotypes (White Perlette, NARC Black, King's Ruby, Sugra One, Vitro Black, and Flame Seedless) were collected from a commercial table grape farm situated in Bhara Kahu, Islamabad, Pakistan (Latitude, Longitude: 33°44'51.8"N, 73°12'10.95"E, Elevation: 588 m) at four distinct stages of development: 20 days after pollination (S1), 30 days after pollination (S2), 40 days after pollination (S3), and 50 days after pollination (S4). To prevent any damage, berries were stored at a temperature of 4 °C.

Ovule culture

The collected berries were thoroughly washed with running water. Subsequently, surface sterilization was carried out by immersing them in 70% ethanol for 2–3 minutes, followed by a 15–20 minute soak in a 1% chlorox solution with occasional shaking. To eliminate any remaining traces of chlorox, the berries were washed with autoclaved distilled water and were placed on autoclaved filter paper for drying. Ovules were removed aseptically form berries and 4–5 ovules/petri plate were cultured on Nitsch and Nitsch medium (30.67 g/L) in combination with 0.5 mg/L GA_3, 30 g/L sucrose and different concentrations of BAP (0.1 mg/L, 0.2 mg/L and 0.5 mg/L) and activated charcoal (1.5 g/L, 2 g/Land 2.5 g/L). Ovules were placed in a growth chamber having temperature of 25–26 °C with relative humidity of 70% and 16/8-hours light. To ensure a continuous supply of nutrients, sub culturing of ovules onto new media was performed every two weeks. After 8 weeks from the initial inoculation, the enlargement of ovules was observed and monitored. Parameters like maximum ovule growth, percentage of enlarged ovules, and percentage of collapsed ovules were recorded after every 2–3 weeks.

Embryo culture

White embryos were detected in certain ovules, and these embryos were subsequently transferred to new media containing 0.5 mg/L Indole acetic acid (IAA). Two weeks following the embryo rescue process, callus induction was observed on some of the culture plates. Sub culturing of calli was carried out every two weeks. During this process, it was noted that some calli turned brown and eventually died, while others exhibited signs of embryo germination.

After two weeks, shoot regeneration was observed in certain calli, and root initiation began two weeks after culturing on new media. Parameters like callus formation percentage and embryo germination percentage were recorded. The seedlings, each possessing four to five leaves, were transplanted into small pots filled with peat moss. After one month, the small plantlets were transferred to larger pots, and they were subsequently placed in a greenhouse to facilitate further growth and development.

Statistical analysis

The experiment was conducted in a three factorial completely randomized block design with four different sampling times and three replications per treatment. Analysis of variance was performed by using R software version 4.1.2, packages (tidy verse, doe bioresearch). Mean comparison was performed with Least Significance Difference test.

Results

Berries of six grapes genotypes collected at four different sampling times were used to investigate the influence of different treatments on the process of embryo rescue. Analysis of variance shows significant differences among all genotypes regarding ovule growth, percentage of enlarged ovules, percentage of collapsed ovules, callus formation percentage and embryo germination percentage (Table 1). Regarding treatments, it was observed that all treatments exhibited highly significant effects on all the measured parameters. The interaction between genotypes and treatments, as well as between genotypes and sampling time, exhibited high statistical significance for all the parameters mentioned. This signifies that the combined effects of specific grape genotypes with particular treatments and sampling times played a substantial role in influencing the results. The heat map illustrates that there are notable variations among the different grape genotypes concerning various parameters across different sampling times and treatments (Fig. 2).

Table 1. Analysis of variance for effect of different sampling time and treatments on Ovule growth, percentage of enlarged ovule, percentage of collapsed ovule, callus formation percentage and embryo germination percentage of different grape genotype.

		OG	EO	CO	CF	EG
Source	df	MSS	MSS	MSS	MSS	MSS
Genotype	5	0.1007**	53.58**	36.5**	21.59**	30.2**
Treatments	8	1.2744**	712.98**	1458.5**	783.73**	1528.3**
Sampling Time	3	51.4089**	8746.74**	23575.1**	9521.83**	15631.7**
Genotype × Treatments	40	0.0410**	2.20**	10**	3.28**	2.4**
Genotype × Sampling Time	15	0.1089**	35.57**	22.5**	7.84**	5.4**
Treatment × Sampling Time	24	0.1125**	132.48**	354**	121.09**	453.4**
Genotype × Treatments × Sampling Time	120	0.0442**	3.23**	12.3**	1.59**	3.3**
Error	432	0.0025	0.79	1.1	0.90	1.2

OG = Ovule Growth; EO = Enlarged Ovule; CO = Collapsed Ovule; CF = Callus Formation; EG: Enlarged Ovule ** = Highly Significant.

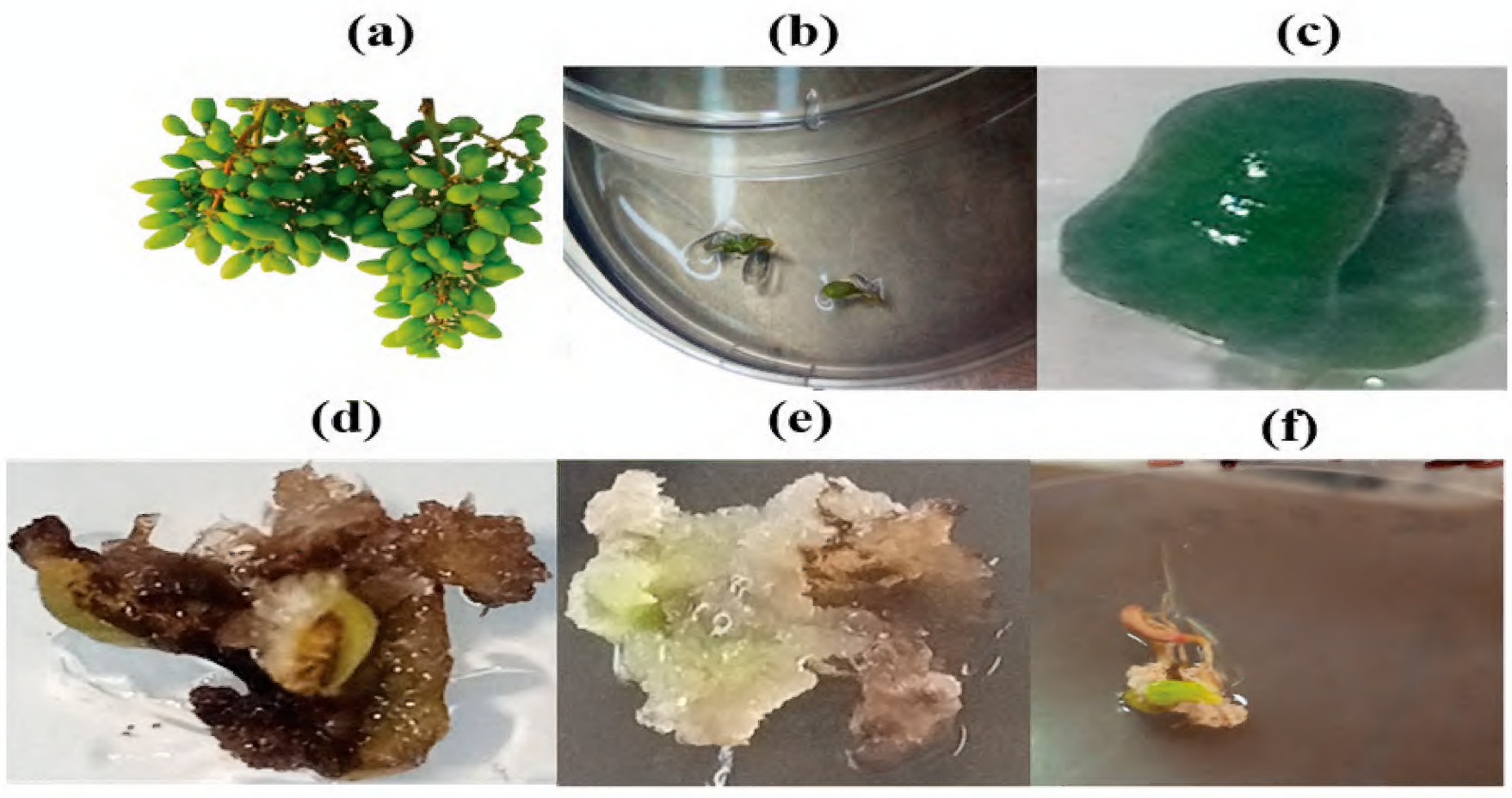

Figure 1. (**a**) Berries collection; (**b**) ovule culturing; (**c**) enlarged ovule; (**d**) collapsed ovule; (**e**) callus formation and (**f**) germinated embryo.

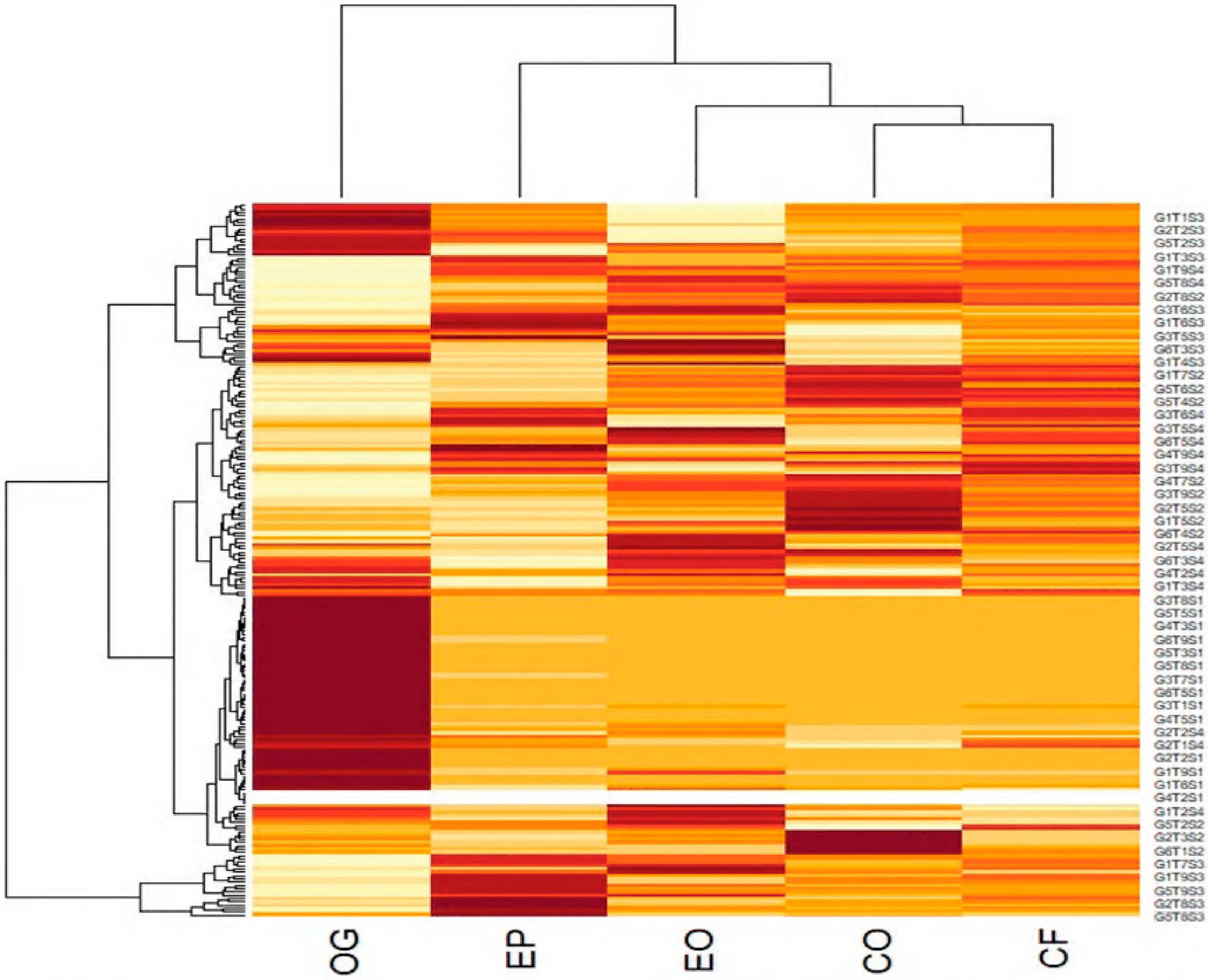

Figure 2. Heat map depicts variations among different genotypes for OG, EO, CO, CF & EP at different sampling time and treatments.

Effect of sampling time and different concentrations of BAP and AC on ovule growth

Maximum ovule growth was recorded 8 weeks after inoculation on culture medium. Maximum ovule growth was observed at treatment 8 having 0.5 mg/L concentration of BAP and 2 g/L Activated charcoal while minimum ovule growth of 0 mm was observed in Flame seedless at treatment 2 (0.1 mg/L BAP, 2 g/L AC) at 20 DAP. Fig. 3A shows that maximum ovule growth of 0.63 mm occurred at treatment 8 (0.5 mg/L BAP, 2 g/L AC) in genotype 4 (Flame Seedless) followed by genotype 3 (Kings Ruby) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) having ovule growth of 0.63 mm. Fig. 3B shows maximum ovule growth of 0.72 mm in genotype 5 (Flame Seedless) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) followed by genotype 5 (Flame Seedless) at treatment 9 (0.5 mg/L BAP, 2.5 g/L AC) having maximum ovule growth of 0.71 mm. According to Fig. 3C maximum ovule growth of 2.93 mm was observed by genotype 5 (Sugra One) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) followed by 2.24 mm by genotype 1 (NARC Black) at treatment 9 (0.5 mg/L BAP, 2.5 g/L AC). Maximum ovule growth of 0.86 mm was observed by genotype 2 (White Perlette) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) as shown in Fig. 3D followed by genotype 3 (Kings Ruby) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) having ovule growth of 0.77 mm.

It can also be concluded that increasing the concentration of BAP and AC in the medium enhances ovule growth. The best results were obtained with treatment 8 (0.5 mg/L BAP, 2 g/L AC), making it the optimal concentration for maximum ovule growth. Results indicate that the optimal sampling time for maximum ovule growth is 40 days after pollination (DAP). It is concluded that 20 DAP is the very early stage for maximum ovule growth followed by 30 DAP, while 50 DAP is the late stage for the growth of ovule. So, 40 DAP is considered as the best sampling time for maximum ovule growth.

Effect of sampling time and different concentrations of BAP and AC on percentage of enlarged ovule

Highest percentage of enlarged ovule of 31.33% was observed at treatment 8 (0.5 mg/L BAP, 2 g/L AC) from berries collected at 40 days after pollination while lowest

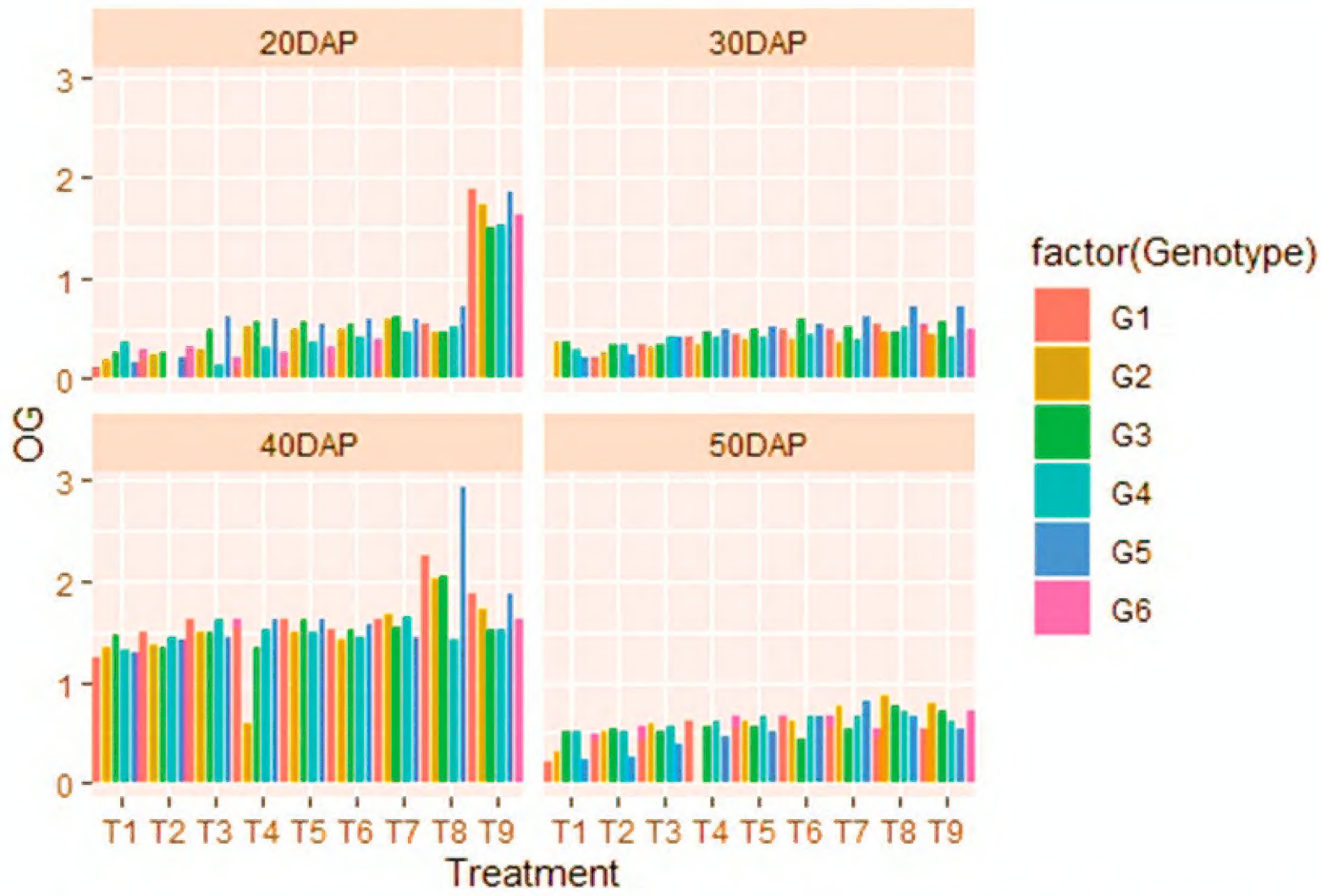

Figure 3. Effect of Sampling Time and Concentrations of BAP & C on Maximum Ovule Growth: (**A**) = 20 DAP, (**B**) = 30 DAP, (**C**) = 40 DAP, (**D**) = 50 DAP.

percentage of enlarged ovule of 0.33% was observed at 20 DAP at treatment 6 (0.2 mg/L BAP, 2.5 g/L AC) followed by 1.66% at 30 DAP at treatment 1 (0.1 mg/L BAP, 1.5 g/L AC). According to Fig. 4A it was observed that maximum percentage of enlarged ovule of 0.66% was observed by genotype 1 (NARC Black) at treatment 5 (0.2 mg/L BAP, 2 g/L AC) and treatment 9 (0.5 mg/L BAP, 2.5 g/L AC), genotype 2 (White Perlette) at treatment 8 (0.5 mg/L BAP, 2 g/L AC), genotype 3 (Kings Ruby) at treatment 7 (0.5 mg/L BAP, 1.5 g/L AC), genotype 4 (Flame Seedless) at treatment 6 (0.2 mg/L BAP, 2.5 g/L AC), genotype 5 (Sugra One) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) and genotype 6 (Vitro Black) at treatment 8 (0.5 mg/L BAP, 2 g/L AC). No ovule showed enlargement at treatments 1 (0.1 mg/L BAP, 1.5 g/L AC), 2 (0.1 mg/L BAP, 2 g/L AC), 3 (0.1 mg/L BAP, 2.5 g/L AC) and 4 (0.2 mg/L BAP, 1.5 g/L AC). Fig. 4B shows that the highest percentage of enlarged ovule of 14% was observed by genotype 3 (Kings Ruby) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) followed by 12.33% by genotype 4 (Flame Seedless) at treatment 8 (0.5 mg/L BAP, 2 g/L AC). Berries collected at 40 DAP (Fig. 4C) showed highest percentage of enlarged ovule of 31.33% by genotype 6 (Vitro Black) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) followed by 29.33% by genotype 4 (Flame Seedless) and genotype 6 (Vitro Black) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) and treatment 9 (0.5 mg/L BAP, 2.5 g/L AC). Highest percentage of enlarged ovule from berries sampled at 50 DAP (Fig. 4D) were observed in genotype 5 (Sugra One) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) having percentage of enlarged ovule of 13% followed by 12.66% at treatment 8 (0.5 mg/L BAP, 2 g/L AC) by genotype 2 (White Perlette). At 50 DAP percentage of enlarged ovule varies from 2–13% among all treatments, so it can be concluded that treatment 8 is considered as best treatment for enlarged ovule. So, by increasing concentration of BAP and activated charcoal in medium there is increase in percentage of enlarged ovule.

Sampling time is also considered as an important factor which plays role in ovule enlargement. According to the above observation it can be concluded that 40 DAP is the best stage for ovule enlargement as less ovule growth occurred at 20 DAP which is the stage at which embryo seems to be not properly developed. So, 40 DAP is the best time for getting a high percentage of enlarged ovule.

Effect of sampling time and different concentrations of BAP and AC on percentage of collapsed ovule

Results revealed that every genotype behaved differently for different treatments regarding percentage of collapsed ovule. Fig. 5A shows the highest percentage of collapsed ovule of 0.66% from enlarged ovule by genotype 1 (NARC Black) at treatment 5 (0.2 mg/L BAP, 2 g/L AC) and treatment 8 (0.5 mg/L BAP, 2 g/L AC) followed by genotype 2 (White Perlette) at treatment 9 (0.5 mg/L BAP, 2.5 g/L AC), genotype 3 (Kings Ruby) at treatment 7 (0.5 mg/L BAP, 1.5 g/L AC), genotype 4 (Flame Seedless) at treatment 6 (0.2 mg/L BAP, 2.5 g/L AC), 7 (0.5 mg/L BAP, 1.5 g/L AC) and 8 (0.5 mg/L BAP, 2 g/L AC), genotype 5 (Sugra One) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) and genotype 6 (Vitro Black) at treatment 8 (0.5 mg/L BAP, 2 g/L AC). No ovule collapsed in all genotypes at treatment 1 (0.1 mg/L BAP, 1.5 g/L AC), treatment 2 (0.1 mg/L BAP, 2 g/L AC), treatment 3 (0.1 mg/L BAP, 2.5 g/L AC) and treatment 4 (0.2 mg/L BAP, 1.5 g/L AC). Highest

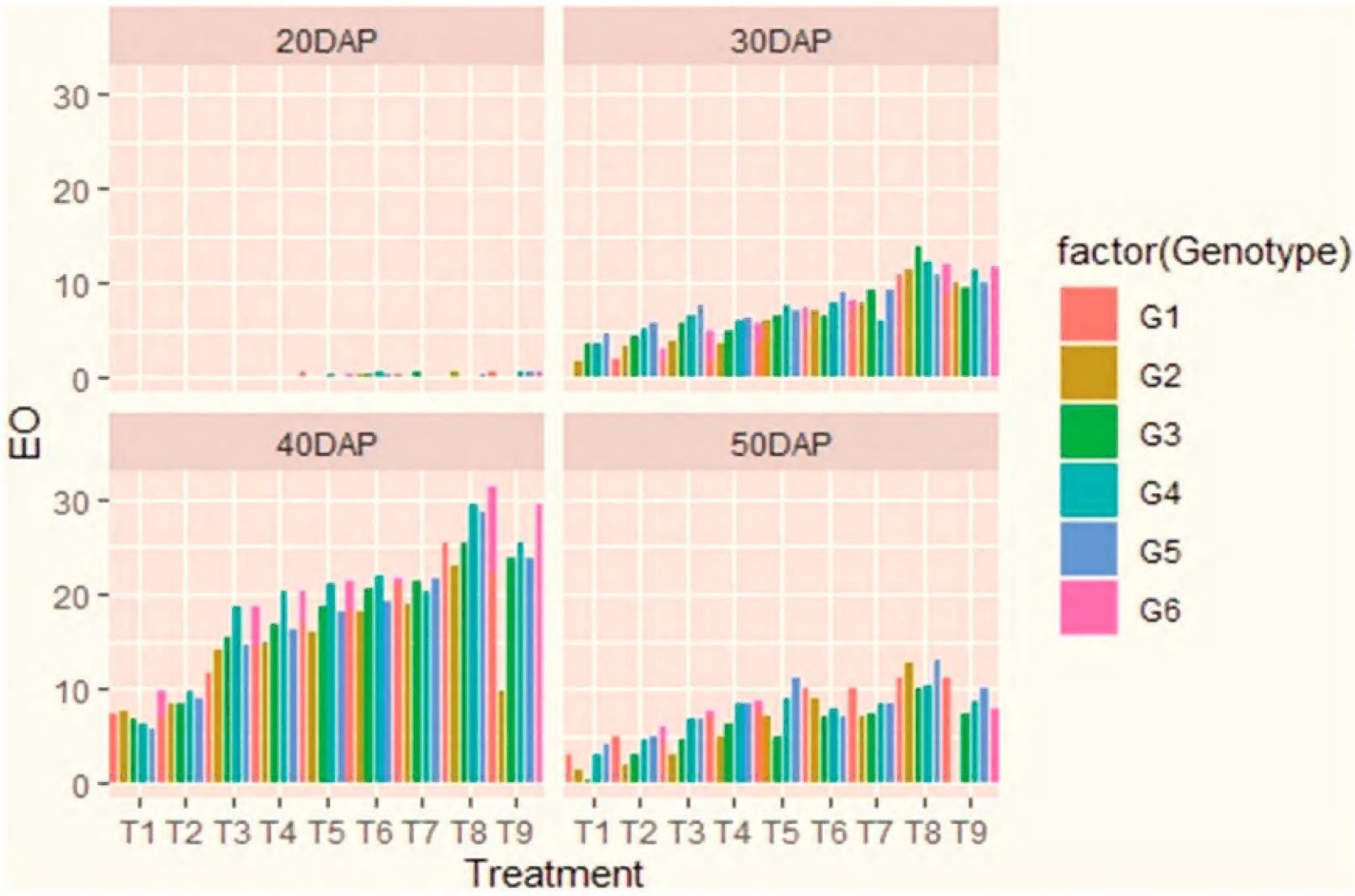

Figure 4. Effect of Sampling Time and Different Concentrations of BAP & AC on Percentage of Enlarged Ovule: (**A**) = 20 DAP, (**B**) = 30 DAP, (**C**) = 40 DAP, (**D**) = 50 DAP.

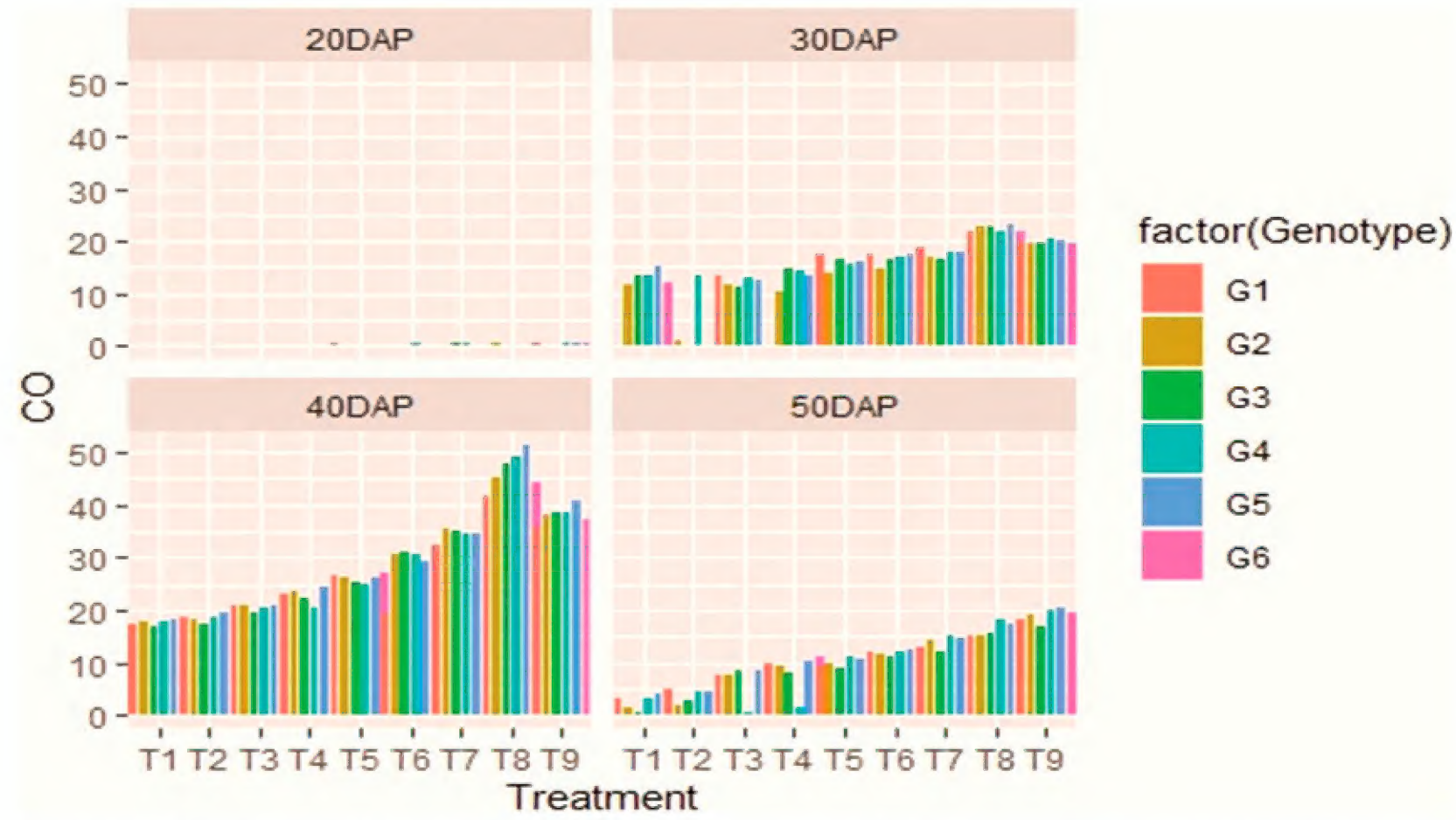

Figure 5. Effect of Sampling Time and Different Concentrations of BAP & AC on Percentage of Collapsed Ovule: (**A**) = 20 DAP, (**B**) = 30 DAP, (**C**) = 40 DAP, (**D**) = 50 DAP.

percentage of collapsed ovule of 23% from enlarged ovule was observed in genotype 5 (Sugra One) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) followed by 22.66% in genotype 2 (White Perlette) and genotype 3 (Kings Ruby) at treatment 9 (0.5 mg/L BAP, 2.5 g/L AC) as shown in Fig. 5B. Fig. 5C revealed that highest percentage of collapsed ovule of 51.33% was observed in genotype 5 (Sugra One) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) followed by 49% in genotype 4 (Flame Seedless) at treatment 8 (0.5 mg/L BAP, 2 g/L AC). The highest percentage of collapsed ovule of 20.33% from enlarged ovule was observed by genotype 5 (Sugra One) at treatment 9 (0.5 mg/L BAP, 2.5 g/L AC) as shown in Fig. 5D followed by 20% in genotype 4 (Flame Seedless) at treatment 9 (0.5 mg/L BAP, 2.5 g/L AC).

The highest percentage of collapsed ovule was observed in treatment 8 (0.5 mg/L BAP, 2 g/L AC) among all genotypes so it can be concluded that treatment 8 is considered to be best for obtaining a higher percentage of collapsed

ovules. Sampling time also has an effect on the percentage of collapsed ovule. Results show that among all sampling time, the optimal time for berry collection to achieve a higher percentage of collapsed ovules is 40 days after pollination.

Effect of sampling time and different concentrations of BAP and AC on callus formation percentage

Results from Fig. 6A shows that highest callus formation of 0.66% occurred in genotype 2 (White Perlette), 3 (Kings Ruby), 4 (Flame Seedless) and 6 (Vitro Black) at treatment 8 (0.5 mg/L BAP, 2 g/L AC), treatment 7 (0.5 mg/L BAP, 1.5 g/L AC) and treatment 9 (0.5 mg/L BAP, 2.5 g/L AC) followed by 0.33% in genotype 1 (NARC Black), 4 (Kings Ruby) and 5 (Sugra One) at treatment 6 (0.2 mg/L BAP, 2.5 g/L AC), treatment 7 (0.5 mg/L BAP, 1.5 g/L AC) and treatment 8 (0.5 mg/L BAP, 2 g/L AC). No callus formation was observed in all genotypes at treatment 1 (0.1 mg/L BAP, 1.5 g/L AC), 2 (0.1 mg/L BAP, 2 g/L AC), 3 (0.1 mg/L BAP, 2.5 g/L AC), 4 (0.2 mg/L BAP, 1.5 g/L AC) and 5 (0.2 mg/L BAP, 2 g/L AC). Fig. 6B shows that the highest callus formation percentage of 22.66% was observed by genotype 3 (Kings Ruby) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) followed by 19.66% by genotype 3 (Kings Ruby) at treatment 9 (0.5 mg/L BAP, 2.5 g/L AC). Highest callus formation percentage of 47.66% was observed by genotype 3 (Kings Ruby) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) followed by 38.66% by genotype 3 (Kings Ruby) at treatment 9 (0.5 mg/L BAP, 2.5 g/L AC) as shown in Fig. 6C. Fig. 6D shows that genotype 3 (Kings Ruby) shows highest callus formation of 17% and 15.66% at treatment 8 (0.5 mg/L BAP, 2 g/L AC) and treatment 9 (0.5 mg/L BAP, 2.5 g/L AC) followed by genotype 5 (Sugra One) and 6 (Vitro Black) having callus formation percentage of 12.66% at treatment 8 (0.5 mg/L BAP, 2 g/L AC).

The result of the data shows that the highest callus formation percentage of 47.66% was observed by genotype 3 (King's Ruby) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) at 40 DAP. Time of appearance of calli is also affected by different concentrations of growth regulators in medium. More calluses appear on medium supplemented with higher concentrations of BAP.

Effect of sampling time and different concentrations of BAP and AC on embryo germination percentage

Results show that the highest percentage of embryo germination was observed by genotype 5 (Sugra One) at treatment 8 (0.5 mg/L BAP, 2 g/L AC). Results of the parameter show that no embryo germination was observed in all treatments at 20 (DAP) as shown in Fig. 7A. Fig. 7B shows that the highest embryo germination of 12% was observed by genotype 2 (White Perlette) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) followed by 11% in genotype 3 (Kings Ruby) at treatment 8 (0.5 mg/L BAP, 2 g/L AC). Fig. 7C revealed that the highest embryo germination percentage of 51% was observed by genotype 5 (Sugra One) at treatment 8 (0.5 mg/L BAP, 2 g/L AC) followed by 50.33% by genotype 4 (Flame Seedless) at treatment 8 (0.5 mg/L BAP, 2 g/L AC). The highest germination percentage of embryo of 15% was observed by genotype 4 (Flame Seedless) at

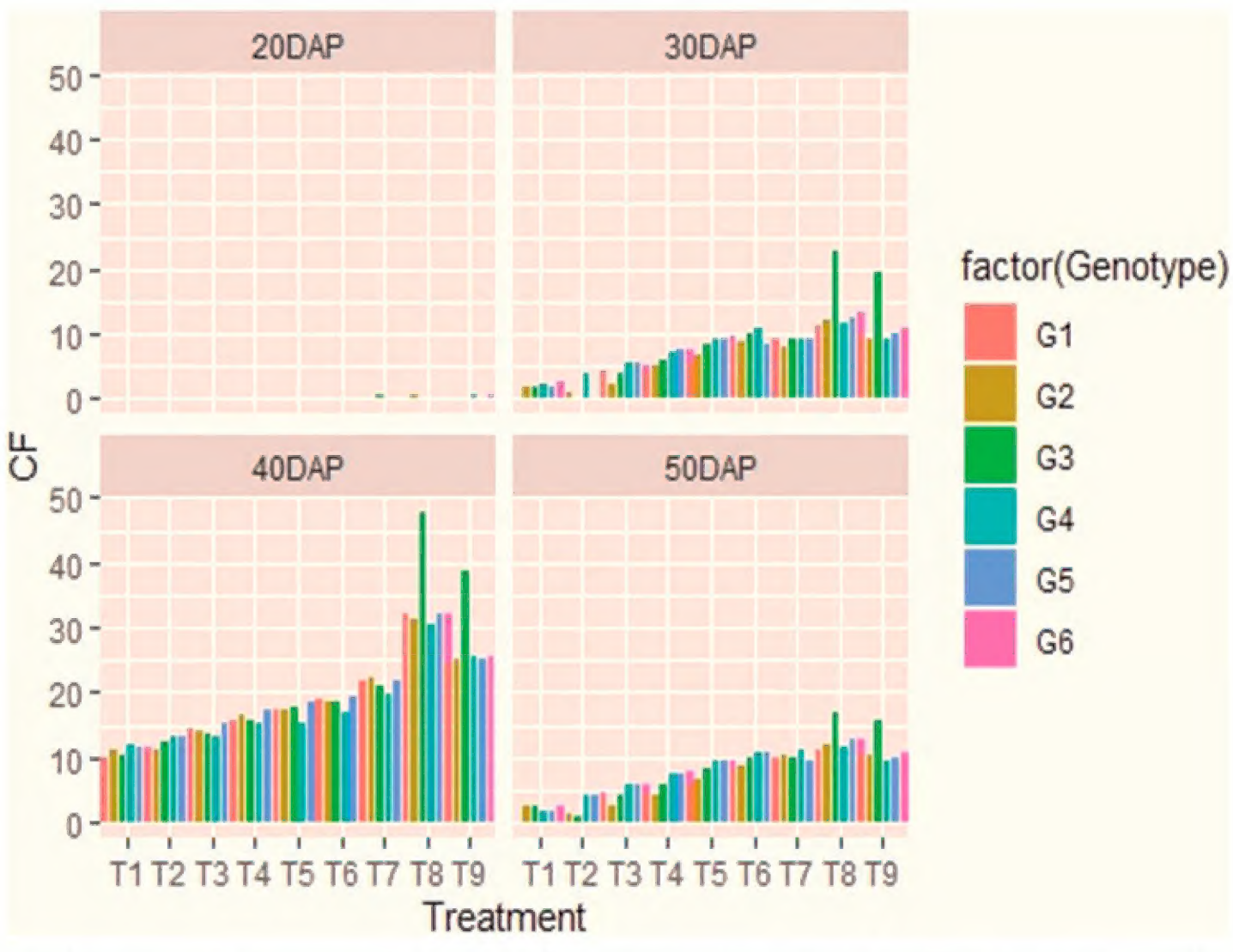

Figure 6. Effect of Sampling Time and Different Concentrations of BAP & AC on Callus Formation Percentage: (**A**) = 20 DAP, (**B**) = 30 DAP, (**C**) = 40 DAP, (**D**) = 50 DAP.

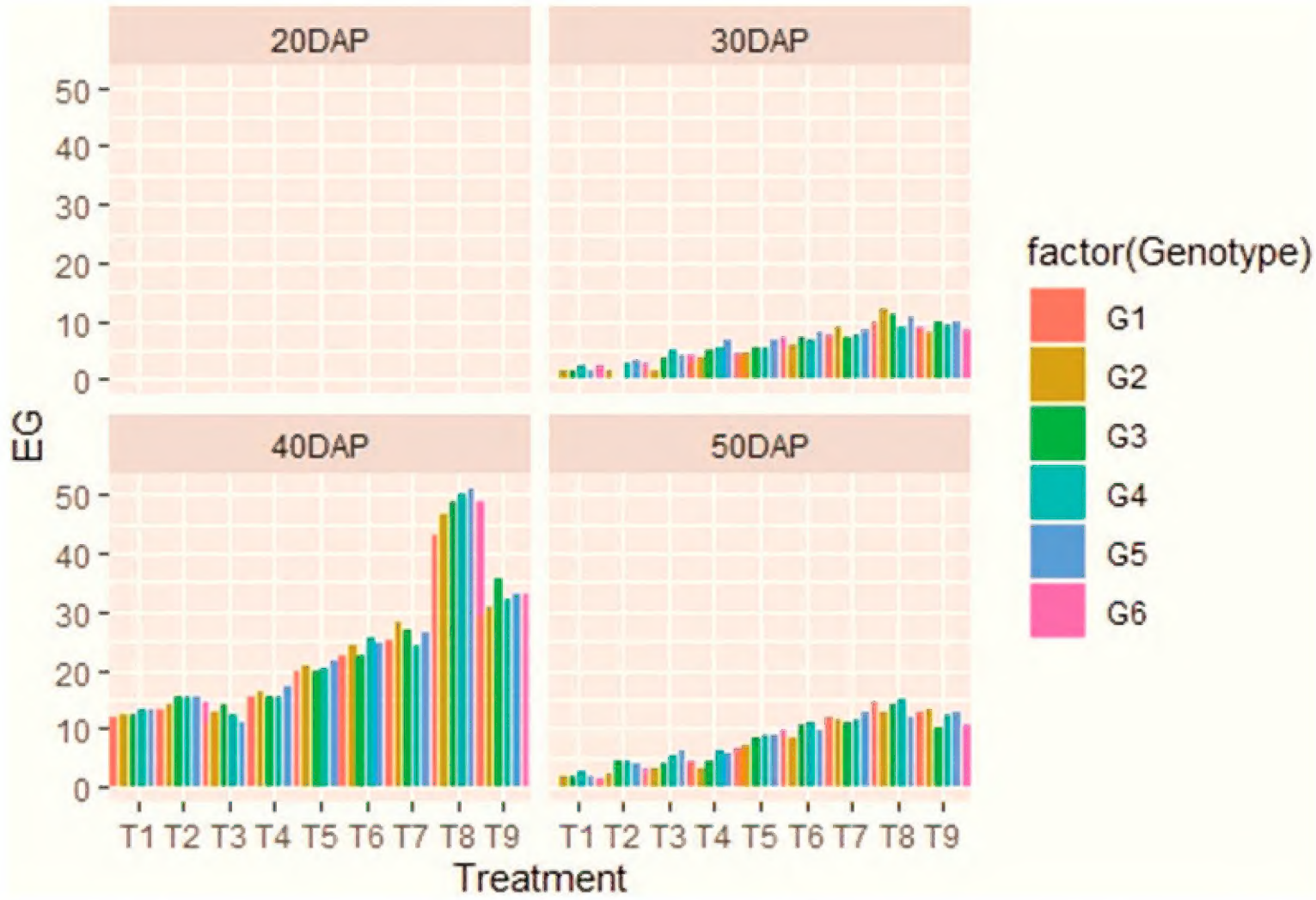

Figure 7. Effect of Sampling Time and Different Concentrations of BAP & AC on Embryo Germination Percentage: (**A**) = 20 DAP, (**B**) = 30 DAP, (**C**) = 40 DAP, (**D**) = 50 DAP.

treatment 8 (0.5 mg/L BAP, 2 g/L AC) as shown in Fig. 7D followed by 14.66% by genotype 1 (NARC Black) at treatment 8 (0.5 mg/L BAP, 2 g/L AC). It was also observed that medium containing low concentrations of AC was not fit for survival of embryo. Embryo survival percentage increased by increasing concentrations of AC in medium. Ovules inoculated on 1.5 g/L of AC resulted in a smaller number of embryos. Less enlargement of ovule and no development of embryos were observed in lowest concentration of AC. Highest concentrations of AC in medium resulted in more development of embryos inside the ovules. It was also observed that by increasing concentrations of BAP in medium survival percentage of embryo increased.

Days after pollination was also another factor in determining the success of embryo rescue. The most suitable time for embryo rescue was determined based on the percentage of ovule enlargement and embryo formation. It was also observed that berries collected at 20 and 30 days after pollination did not develop viable embryos as embryos were not properly developed at this stage. So, it is difficult to save embryos at this stage due to their inadequate development.

Discussion

Immature berries of six grapes genotypes were collected at different days after pollination to check the effect of different sampling time and different concentrations of BAP and Activated charcoal on ovule culture and embryo rescue. Data for the following parameters i.e. ovule growth, percentage of enlarged ovule, percentage of collapsed ovule, callus formation and embryo germination percentage were recorded. The findings indicated that, among all the treatments, treatment 8 (consisting of 0.5 mg/L BAP and 2 g/L AC) exhibited a favorable response towards all measured parameters. Observations were also correlated with Oláh (2017) that presence of activated charcoal in the medium creates a darker environment, which in turn reduces browning by minimizing the presence of inhibitory substances such as ethylene and phenolic compounds. This, in turn, contributes to enhancing plant development. Activated charcoal has a very fine network of pores so it has the ability to absorb many substances in it. In tissue culture media, its primary impact is on morphogenesis, achieved through the adsorption of inhibitory compounds thus reduces the presence of toxic metabolites and contributes to the mitigation of browning (Thomas 2008; Wang et al. 2022). Similar results are also reported by Singh et al. (1990) that 0.5 mg/L BAP gives maximum ovule growth. By increasing the concentration of activated charcoal in medium reduction in browning of calli were observed. Results also correlate with Amente and Chimdessa (2021), that activated charcoal in medium proved to be very effective to minimize the issue of browning. Results are in accordance with Oláh (2017). So a concentration of 2.5 g/L of activated charcoal in the medium has been found to contribute to ovule enlargement as well. It was observed from experiments that maximum ovule growth occurred in samples collected at 40 days after pollination (DAP). These results align with the findings of van Tuyl and de Jeu (2009), who similarly reported that the highest ovule growth was observed when sampling was conducted at 42 DAP.

Regarding highest percentage of enlarged ovules, results are in consistent with Gray (1992) that by addition of BAP in medium led to a higher percentage of enlarged ovules and greater somatic embryo development. Furthermore,

the results proved that 40 days after pollination (DAP) is the optimal time for obtaining a high percentage of enlarged ovules. Results are in accordance with Khoshandam et al. (2017) who observed that highest ovule enlargement occurred at 45 days after pollination. Similar results are also reported by Guimei and Hanfeng (2001) that more ovule development was observed when samples collected at 35–49 DAP. Results were also in accordance with Ismail and Khalil (2021) who also identified the best time for embryo rescue in ovule culture. Highest percentage of collapsed ovule was also observed at treatment 8 on sampled collected at 40 DAP. Ebadi et al. (2016) also reported that the addition of activated charcoal in the medium and a sampling time of 45 days after pollination (DAP) prove to be optimal for embryo germination and subsequent plant development, particularly in Flame Seedless cultivars. Giancaspro et al. (2022) reported that highest number of fertilized ovules were obtained from berries collected at 30 DAP followed by 40 DAP.

The time of callus formation is influenced by the concentrations of growth regulators in the medium. Specifically, a higher concentration of BAP in the medium tends to promote the appearance of more calli. Mahadi et al. (2016) and Cui et al. (2013) also reported that an increase concentration of growth regulators such as 2,4-D and BAP in the medium has a positive effect on callus induction, resulting in the production of a greater number of calli within a shorter period of time. Similar results are also reported by Singh and Brar (1993), that maximum callus induction was observed at 0.5 mg/L BAP. Xu et al. (2022) also did ovule culture of grapes by inoculating ovules on different medium and also observed sampling time for ovule culture. Results showed that more callus induction occurred in samples collected at 40 DAP. Similar results are also reported by Gray et al. (2019) that best sampling time for ovule culture is 42 days after flowering. Haiying and Ailing (2005) determined the appropriate sampling time for grapes ovule culture and used early maturing, middle maturing and late maturing grapes cultivars reported that best sampling time for early ripening cultivars is 42 DAP. Teng-Fei et al. (2022) also reported best sampling time for efficient embryo rescue of different grape genotypes.

The result shows that the highest percentage of embryo germination was observed by genotype 5 (Sugra One) at treatment 8 (0.5 mg/L BAP, 2 g/L AC). It was also observed that medium containing low concentrations of AC was not fit for survival of embryo. Embryo survival percentage increased by increasing concentrations of AC in medium. Similar results were observed by (Valdez 2005) by the use of 2.7 g/Lof AC in NN medium. He also observed that AC in medium promotes embryo culture and formation of zygotic embryos. Results were also similar with (López-Pérez et al. 2005) who used AC in medium and observed that AC in medium help in development of somatic embryos and also increase the f requency of embryogenic calli. Results were also correlated with (Singh et al. 1990) who also had similar observations at medium containing 0.5 mg/L BAP. Results were also similar with (Benke et al. 2021) who also reported increase in embryo germination by increasing concentration of BAP in medium. Maximum embryogenesis of calli were also observed by (Alavijeh et al. 2016) by increasing concentrations of BAP in medium. Results were quiet similar to (Yang et al. 2007) who reported that best stage for embryo development is 40–45 days after pollination. Similar results were reported by (Huang et al. 1991). He observed that the time of sampling plays a significant role in embryo development. If the sampling time is too late, it becomes challenging to rescue the embryo at a later stage, often resulting in embryo abortion. Findings were aligned with (S. Li et al. 2020) who reported that higher embryo germination occurred in samples collected at 37–42 days after pollination. Similar results were also reported by (Razi et al. 2013) who also noted that the optimal sampling time for achieving the highest embryo germination rate was at 40 days after pollination.

Conclusion

The study highlights the crucial role played by different sampling times and concentrations of BAP (Benzyl aminopurine) and AC (Activated Charcoal) in optimizing the protocol for efficient embryo rescue in grapes. The results indicate that the highest number of ovules and the best germination percentage were observed in samples collected at 40 days after pollination (DAP) when inoculated on a medium containing 0.5 mg/L BAP and 2 g/L AC. Additionally, the presence of some seed traces in the samples influenced the optimal time for embryo abortion, which was recorded at 50 DAP. However, the best time for successful ovule culture followed by embryo rescue was found to be at 40 DAP. Therefore, based on findings, it is recommended that 40 DAP and the combination of 0.5 mg/L BAP and 2 g/L AC be considered as the appropriate sampling time and the best hormonal combination for efficient grape embryo rescue. These insights can be valuable for grapevine breeding and the development of disease-resistant seedless grape varieties.

Author Contributions

MH conceptualized the idea for research. QN conducted the experiment and collected data. MJA and HSA collected reviews of literature and helped in funding. RMR and IA prepared the original draft of the manuscript. All the authors participated in the review and editing process to refine the paper before submission.

Conflicts of Interest

The authors declare no conflict of interest.

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Data Availability Statement

Available upon request from the corresponding author.

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